'The genetic analysis of functional connectomics in *Drosophila*'

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ABSTRACT

Fly and vertebrate nervous systems share many organization characteristics, such as layers, columns and glomeruli, and utilize similar synaptic components, such ion channels and receptors. Both also exhibit similar network features. Recent technological advances, especially in electron microscopy, now allow us to determine synaptic circuits and identify pathways cell-by-cell, as part of the fly's connectome. Genetic tools provide the means to identify synaptic components, as well as to record and manipulate neuronal activity, adding function to the connectome. This review discusses technical advances in these emerging areas of functional connectomics, offering prognoses in each and identifying the challenges in bridging structural connectomics to molecular biology and synaptic physiology, thereby determining fundamental computation mechanisms that underlie behaviour.

I. INTRODUCTION

A century after Cajal compiled his comprehensive catalogue of cell types in the vertebrate brain (Cajal, 1909, 1911) the neuron doctrine for which he was such a vocal champion survives as received dogma, but is increasingly supplanted by a view of the nervous system that emphasizes the latter's properties as a functional network (Bullock *et al*., 2005; Grillner, 2006). The search for neural networks is of course nothing new, only now made possible by new digital, imaging and computational technologies that confront with sufficient force the problems presented by the brain's intractable features. Five of these have been widely recognized: the diversity of cell types in any nervous system; the problems of imaging neural activity on a millisecond timescale; the physical dimensions of neurons (their local dimensions at synapses and their long reach within the conducting pathways of the brain); the fact that synaptic contacts between neurons cannot usefully be resolved by light microscopy within the brain's depth; and finally the requirement to reconstruct comprehensively all connections between different populations of neurons in order to resolve all the pathways between them (Lichtman and Denk, 2011).

A key issue in the search for the comprehensively reconstructed networks of a nervous system, its connectome (Sporns *et al*., 2005; Lichtman and Sanes, 2008), is the one of resolution. How accurately do we need to reconstruct synaptic circuits in order to understand their function? It is often argued that motor systems in simple brains with few neurons, such as are found in many invertebrates, might rely on connections that are highly specific because in a simple system a faulty connection is likely to be lethal. On the other hand, the multiple parallel pathways of a sensory system, such as those that underlie the fly's compound eye, in fact incorporate very few projection errors (Horridge and Meinertzhagen, 1970; Meinertzhagen, 1972). In contrast, the large brains of, for example, vertebrates and cephalopod molluscs are often thought to utilize only largely stochastic signals (e.g. Jazayeri and Movshon, 2006), for which connections between interneurons presumably need only be statistical. On the other hand, another view emphasizes the centrality of the brain's exact connectome (Seung, 2012).

Resolving these different views poses a major technical challenge, one solution to which is to concentrate on the numerically simple nervous systems of genetically manipulable organisms. Even in those cases, reconstructing a neural circuit at the ultrastructural level so as to resolve its complete network of connections is a painstaking process, one that is complete only in the entire nervous system of *C. elegans* (White *et al*., 1986), whose simple tubular neurons are well suited to such comprehensive analysis. In other species, the same goals have been restricted to parts of nervous systems with few neurons, such as the optic lamina neuropiles of isogenic *Daphnia magna*, the water flea (Macagno *et al*., 1973; Sims and Macagno, 1985) or the fruit fly *Drosophila melanogaster* (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Rivera-Alba *et al*., 2011). In these cases, the connections between identified neurons appear to be highly specific, with some variation in the branching patterns and synapses of the same cells in isogenic *Daphnia* (Macagno *et al*., 1973), but relatively little in *Drosophila* (Meinertzhagen and Sorra, 2001; Rivera-Alba *et al*., 2011).

Recent technical advances in image acquisition and processing have already begun to accelerate progress in circuit reconstruction (Kleinfeld *et al*., 2011), and to consolidate the new field of connectomics (Sporns *et al*., 2005) at single-cell level. Like genomic sequences, structural connectomes generated by these projects provide a foundation upon which to build functional data, from the patterns of gene expression to detailed synaptic properties. Here we refer to such meta data sets as a functional connectome. Just as genes are the building blocks of genomics, neurons are the key units of functional connectomics. In the same way that parsing genomic functions requires information on gene expression patterns, interactions

among genes, and the kinetic properties of those interactions, so understanding how neural circuits function requires explicit and comprehensive information on neural activity, synaptic connections and synaptic properties.

As recognized by early advocates (e,g. Rubin, 1988; Miklos, 1993) *Drosophila* has proved a powerful resource in the discovery of genes required for nervous system development and function. More recently, *Drosophila* neurobiology has begun to shift, however, moving from gene-centered to neuron-centered approaches. Numerous genetic tools have been developed to monitor neuronal activity and target its manipulation by means of the Gal4/UAS and related systems (Simpson, 2009). *Drosophila* provides particular opportunities for conditional expression especially using temperature-sensitive (ts) alleles of genes for synaptic proteins or ion channels. The clearest example is a UAS construct incorporating the shi^{ts1} allele of the gene *shibire* coding for dynamin (Kitamoto, 2001), which induces synaptic blockade at the non-permissive tempature, albeit at the cost of low background expression that may compromise cell integrity (Gonzalez-Bellido *et al*., 2009). These techniques, especially using UAS-shi^{ts1}, allow us to dissect neural circuits and identify the functional roles of specific neurons within them, as defined by behavioural outcomes from their modified or failed transmission (e.g. Rister *et al*., 2007; Gao *et al*., 2008), or as read out from the responses of downstream neurons (e.g. Schnell *et al*., 2012).

From a broader perspective, *Drosophila* has unique contributions to make to the connectomic analysis of model nervous systems. Despite its small size, the fly's brain shares various organizational features with the nervous systems of vertebrates, in particular in its subdivision into layers, columns and glomeruli. Numerous parallels have been drawn, especially for the olfactory systems of different groups (Hildebrand and Shepherd, 1997). Aside from such advocacy statements, *Drosophila* has the powerful advantage that its neurons can be uniquely identified based on their morphological determinacy, gene expression patterns and synaptic connections (Meinertzhagen *et al*., 2009). These qualifications, linked to the opportunities provided by Gal4-targeted effector reagents (Simpson, 2009) make *Drosophila* an ideal species in which to attain the goal of functional connectomics -- linking its structural connectome to synaptic and circuit physiology.

In this review, we attempt to cover two areas: current progress in determining the synaptic connections in neural circuits of the fly's brain; and assigning functional synaptic components to specific connections within the circuits. Other aspects of functional connectomics, such as imaging (e.g. Riemensperger *et al*., 2012) and manipulating (Simpson, 2009) neural activity, have been well reviewed elsewhere and will only be updated here. Given that this is a relatively new field, we will include not only the methods used to trace synaptic circuits in the fly but also those originally described in other systems with potential *Drosophila* applications. We will highlight the advantages of different techniques and their potential pitfalls. Finally, we will discuss the challenges and prospects for functional connectomics in *Drosophila*.

II. REVEALING THE STRUCTURAL CONNECTOME

A. Reconstructing synaptic circuits by electron microscopy

Electron microscopy (EM) is, in our view, the sole means to identify the exact composition of synaptic contacts between identified neurons in the brain of *Drosophila*, and is thus essential in the analysis of the fly's connectome (Table 1). From current evidence, sites of chemical synaptic transmission have an average packing density in the fly's brain of about two per cubic micron, for example 2.74 for the mushroom body calyx (Butcher *et al*., 2012). Active zones are often revealed by the presence of a presynaptic dense body (Atwood *et al*., 1993) or ribbon (Fröhlich and Meinertzhagen, 1982), T-shaped in cross section (Prokop and Meinertzhagen,

2006). T-bar ribbons are typical of all anatomical synapses in the visual system but not necessarily all elsewhere, constituting only some of the contacts in the mushroom body calyx of the olfactory system for example (Yasuyama *et al*, 2002; Butcher *et al*., 2012) or the lateral horn (Yasuyama *et al*., 2003). Non-ribbon synapses have a simple presynaptic density at the plasma membrane, often large in area, similar to that seen at the neuromuscular varicosities (Atwood *et al*., 1993). Compatible with their function during transmission, T-bar ribbons often lie beneath a population of synaptic vesicles, but these are not always focal; they may fill much of the entire presynaptic terminal, for example, as they do at many synapses in the visual system. Although neuromuscular (Atwood *et al*., 1993) and giant fibre (Blagburn *et al*., 1999) synapses provide input upon a sole single postsynaptic element, synapses of the central nervous system each site are usually polyadic, with multiple postsynaptic contacts, about four for the fly's medulla neuropile (Takemura *et al*., 2008) but up to a dozen in the mushroom body calyx (Butcher *et al*., 2012).

How do we know that networks formed by structural synapses are actually functional? Direct evidence is based mostly on neuromuscular and photoreceptor synapses. Focal recordings from neuromuscular varicosities establish the close correlation between active zones and the strength of transmission (e.g. Stewart *et al*., 1996). At the lamina's photoreceptor tetrads light-exposure results in vesicle exocytoses beneath the T-bar ribbon (St Marie and Carlson, 1982). Connections with many structural synapses also constitute relays between neurons that constitute functionally identified pathways, such as from Mi1 to T4 in the proximal medulla (Takemura *et al*., unpublished). Anomalies exist, however. Input from photoreceptor R8 to R7 is suggested by the presence of many preynaptic T-bar ribbons, but denied by the lack of expression of the histamine receptor reporter *ort* (Takemura *et al*., 2008). A medulla neuron, T1, receives synaptic inputs but lacks structural evidence of output synapses (Takemura *et al*., 2008), possibly providing such output via gap junctions, however.

The major problem in identifying circuits lies of course not in identifying their sites of synaptic contact, despite the problems that this alone may pose, but in tracing both pre- and postsynaptic sites back to an identified cell type, because only then can circuit information be derived. But the conceptually simple task of identifying profiles of the same neurite in consecutive images is technically difficult, especially for the fine arborising dendrites of *Drosophila* neurons. Although these differ from the dendrites of vertebrate neurons in being located away from the soma, they share many features in common, even if they do not invariably lack presynaptic sites as sometimes claimed (Sánchez-Soriano *et al*., 2005). Presynaptic sites are however concentrated at the terminals of neurons, which contain mitochondria and presynaptic organelles and are therefore generally stouter in calibre, but terminals need not be exclusively presynaptic, any more than dendrites are restrictively postsynaptic (Takemura *et al*., 2008). So, in neither case can the partition of synaptic territories between dendrite and terminal be explicitly assumed. Reflecting this flexibility in synaptic roles, relay circuits incorporating projection neurons are augmented by richly interconnected local microcircuits. For example in the lamina (Meinertzhagen and O'Neil, 1991), up to 40% of synapses contribute to the latter (Meinertzhagen and Sorra, 2001).

Serial-section EM (ssEM) is the longest standing method for tracing neurites in the neuropile ('tracing wires': Denk *et al*., 2012) and neuropile depth in the small brain of *Drosophila*, which rarely exceeds 50 μm for any single compartment, partially offsets the technical disadvantages of ssEM in this species. Limited success has in fact already been achieved, for example in the visual system (Sprecher *et al*., 2011) and neuromuscular junctions (Atwood *et al*., 1993) of the larva, and in the adult brain, for example in the medulla (Takemura *et al*., 2008) and mushroom body calyx (Butcher *et al*., 2012). New semi-automated procedures (e.g. Chklovskii *et al*., 2010) now secure limited success using ssEM approaches to reconstruct the three-dimensional

shapes of neurons, but still require labour-intensive human checking of profile continuity, which is currently the rate limiting step to obtaining complete circuit information. Achieving a successful trace is still particularly difficult in the case of the postsynaptic dendrites, which in *Drosophila* are especially fine and delicate, often bordering on the dimensions of the thickness of the very ultrathin sections used to visualize them, around 50nm. Neurites frequently change direction in the neuropile, and are of such small calibre as to be mostly included within the thickness of a section, so that if they travel sideways for any distance they are often lost.

In view of these difficulties, increasing attention has recently been directed to alternative approaches to ssEM. Two such methods in particular are serial block-face scanning electron microscopy (SBFSEM; Denk and Horstmann. 2004), and focused ion-beam (FIB) milling of specimen blocks (Knott *et al*., 2008, 2011; Table 1). Both have the advantage that they leave the specimen intact prior to imaging, and thus produce an image stack that is pre-aligned. SBFSEM has been used successfully to reconstruct the shapes of vertebrate neurons (e.g. Briggman *et al*., 2011), but requires methods to enhance membrane contrast that can make it difficult to attain the correct compromise between tracing neurites and identifying synapses. The spatial resolution of this method has been reported as 10 nm in x and y but 23 nm in z (Briggman *et al*., 2011), also not well suited to the more delicate and densely branched neurons of *Drosophila*. On the other hand, the use of FIB seems far better qualified, but not so far reported, for *Drosophila*. The chief advantage lies in the improved z-axis resolution, which can be matched to that in x and y, so as to generate an image stack of isotropic voxels typically at a resolution of 5-10nm for acceptable rates of image capture (Hess and Xu, pers. comm.). The voxels can then be resectioned to yield virtual images in x,z and y,z as well as in oblique planes; available evidence indicates that these provide the basis for improved accuracy and speed in tracing neurites (Rivlin *et al*., pers. comm.). FIB's chief disadvantage lies in the limited volumes that can be routinely imaged. These methods and their respective advantages have recently been compared (Denk *et al*., 2012, Table 1) and in the case of FIB in particular are under active development for work on *Drosophila*.

By contrast with chemical synapses, the distribution of electrical synapses (gap junctions) is not well characterized in the *Drosophila* brain (Bauer *et al.,* 2005). Sites of membrane apposition are identified in specific locations where coupling is known to exist, for example between terminals of the giant fibre axon (Blagburn *et al*., 1999) or of photoreceptors R1-R6 in the lamina (Shaw and Stowe, 1982; Shimohigashi and Meinertzhagen, 1998), but insufficiently distinct to recognize reliably or comprehensively elsewhere. As a result, no reliable estimate appears to exist for the total numbers of gap junctions in any single brain region. Systematic studies on the regional expression of the *innexins* (*inx*) that encode gap-junction proteins in protostomes (Phelan *et al*., 1998), with eight member genes in *Drosophila* (Phelan and Starich, 2001; Phelan, 2000, 2005), all but *inx4* of which express in the developing CNS of the 50% pupa (Stebbings *et al*., 2002), are still required at the cellular level (Table 2.2). Heteromerisation (channels comprising different subunits) is common, leading to intercellular channels of homotypic composition (two hemichannels identical) or heterotypic (two hemichannels of differing molecular composition) (Lehmann *et al*., 2006). The latter in turn will require evidence for which different Innexins localize to coupled cells. Freeze-fracture methods (e.g. Chi and Carlson, 1980; Shaw and Stowe, 1982) provide clear evidence of the particular arrangements at such junctions, but frustrate the identification of most neurons. Innexin reagents that could localize gap junction protein expression to the membranes of cells known from ssEM to contact each other would seem currently to provide the best avenue to localize gap junctions.

B. Labelling specific neurons with electron-dense markers

The requirement either to reconstruct the three-dimensional shape of a neuron or simply identify the profiles it contributes to a single section can in theory be met by labelling the neuron in

question with an electron-dense marker. Currently the best marker for that purpose is the enzyme horseradish peroxidase (HRP) whose action on peroxides can be visualized in the EM by the electron donor diaminobenzidine (DAB) when it is rendered osmiophilic (Graham and Karnovsky, 1966). When HRP is targeted to specific organelles, such as the plasma membrane, for example by means of the Gal4-UAS system used in conjunction with UAS-mCD2::HRP (Larsen *et al*., 2003), specific Gal4 drivers can be used to label specific neurons (Clements *et al*., 2008; Edwards and Meinertzhagen, 2009) and the results compared with the light microscopic expression of green fluorescent protein (GFP) under control of the same driver. Sound in principle, this method is limited by the strength and specificity of the driver, by the prolonged incubation in DAB that can be required and that may compromise ultrastructural preservation, by the limited diffusion of reagents (Clements *et al*., 2008), and by mosaicism of the label. Moreover, the intensity of the electron-dense signal usually varies, partly because of the section plane, but also regionally, possibly as a result of local membrane turnover. It is also theoretically possible that inserting excessive amounts of HRP into the membrane can alter membrane surface function, leading to the spectre of induced changes in connectivity. Nevertheless, this approach has proved useful to identify the slender postsynaptic dendrites of medulla cells (Gao *et al*., 2008), and is sufficient to identify synaptic connections, provided the corresponding presynaptic terminal can be recognized by ultrastructural, shape, or positional criteria. Alternatively, in a further development of existing methods, it would be advantageous to generate genetic methods that also label the presynaptic terminal in the same fly, for example by independently targeting HRP to intracellular organelles, such as the mitochondria that cluster presynaptically. This would then enable pre- and postsynaptic partners to be labeled in the same preparation if the expression of HRP were roughly matched between those partners so as to visualize both after the same DAB incubation. Such double-label methods will require the further development of reagents and have yet to be reported.

C. Assessing the diversity and identities of cell types

Many neurons in the fly's brain have been identified from previous studies by means of light microscopy (LM). Most have been identified in the glomerular -- as opposed to diffuse -- neuropiles (Hanström, 1928), in which the neuropile is subdivided into modules readily seen and identified by light microscopy. In fact, we can imagine that most neuropiles have an organisation that is repeated, their constituent neurons having determinate locations and connections, but that only in some is this organization revealed at the light microscopic level by the repeated arrangement of recognizably larger neuron profiles or distinctive neuronal groups. The optic lobe provides a case in point. The most distal neuropile -- the lamina -- is clearly modular, with an array of cartridges that corresponds to the overlying array of ommatidia in the compound eye, and this modularity is still obvious in the distal strata of the next neuropile -- the medulla, but becomes far less so in the third neuropile, the lobula (Meinertzhagen, 2012).

Early reports from Golgi impregnation in *Drosophila* (Fischbach and Dittrich, 1989; Hanesch *et al*., 1989) and other fly species (e.g. Strausfeld, 1976, 1980) reveal the range and diversity of cell types. The library of cell types compiled by this method presents two main uncertainties: whether it contains all cell types and whether these are discrete, each type different from all the others. These issues have been discussed (e.g. Fischbach and Dittrich, 1989; Strausfeld, 1980) but are in the end determined empirically, using alternative methods to reveal the same neuron types and numbers repeatedly in different samples by different methods.

A certain amount of morphological variation occurs within each neuron type, especially among the furthest dendrites. This variation is clearly discernable among the morphologically determinate neurons of the visual system (Lee *et al*., unpublished) but is distressingly greater in the olfactory system (e.g. Marin *et al*., 2002; Chou *et al*., 2010). Cluster analyses provide a powerful means to differentiate cell types (e.g. McGarry *et al*., 2010), but have yet to be applied

to *Drosophila* neurons, which moreover support alternative genetic approaches to the determination of cell types. So, while morphometric analyses support the notion that cell types are discrete, but with discernable morphological variations within each type (Marin *et al*., 2002), conclusive evidence has come from the genetic reporters that now supplant classical methods as the means to identify cell types in *Drosophila*.

Where the neuropile is arranged in columns and strata, as for the optic neuropiles, cell types have been distinguished along morphogenetic grounds into columnar, tangential and amacrine (Fischbach and Dittrich, 1989), depending on the primary direction of growth of the axon. Each type is identified by taxonomic criteria devised by a human observer, based largely on the number, shape and stratum of its arborization in the neuropile, and for the types observed in the optic lobe (Fischbach and Dittrich, 1989) each class has been reasoned to be discrete and its representatives isomorphic. From this evidence alone, however, there is no guarantee that all types have been reported, nor that all subtypes identified by the human observer are in fact real, nor that individual types may not comprise additional subtypes. The latter is for example true for cell Tm5, now known to have three forms, a-c (Gao *et al*., 2008). Thus, no independent assessment of the number of types exists to arbitrate these uncertainties, even though many cells have now also been seen using genetic reporters. The latter are now available in great number in *Drosophila* and clearly supersede evidence from Golgi studies. Reporter lines have been isolated from screens undertaken in two locations in particular. The Ito group in Tokyo have reported lines for adult auditory (Kamikouchi *et al*., 2006) and visual projection neurons (Otsuna and Ito, 2006), mushroom body (Tanaka *et al*., 2008) and antennal lobe-associated neurons (Tanaka *et al*., 2012), and has established an online database that compiles information on these (Shinomiya *et al*., 2011). In addition, an extensive library of neural cell profiles aims to identify every class of neuron in the fly's brain (Chiang *et al.,* 2011). At the Janelia Farm campus of HHMI, the group of Rubin used a preliminary strategy to generate more than 5000 lines from enhancer driven expression of Gal4 in subsets of 50-100 cells (Pfeiffer *et al*., 2008). This strategy was based on initial estimates for the typical numbers of cells per cell type, and the lines generated have indicated that each expresses on average in about 15 cell types (Rubin, pers. comm.). As part of an intersectional strategy to refine the pattern of expression yet further, the initial lines are next being combined to drive LexA::VP16 reagents (Lai and Lee, 2006) as a second expression system, and thus divide the pattern of expression for each line into even smaller subsets of cells (Pfeiffer *et al*., 2010). It seems reasonable to expect that eventually sufficient lines will become available to label each and every cell type of the fly's brain as a single class.

To examine the morphology of individual cells representative of a single type within a particular expression pattern, various methods are available, such as MARCM and flip-out techniques (Lee and Luo, 1999; Wong *et al*., 2002) to generate clones of labeled neurons. Targeting the fluorescent label to the plasma membrane of specific neurons, for example using UAS-mCD8-GFP under the control of an appropriate Gal4 line (Lee and Luo, 1999), yields images of cells that in many cases compare closely to, and are then often named after, Golgi impregnates. A database of cell types has been established (Shinomiya *et al*., 2011). An independent genetic nomenclature has yet to be developed. Subtle manipulations of the numbers of cells can be made by the dosage of transposase, and in some cases the labeling pattern of each strain is largely the same, while in a few cases the labeling pattern may also vary with the particular UAS reporter line (Ito *et al*., 2003). It is therefore satisfying that, at least in select cases, neurons such as medulla cell Tm2 originally described from Golgi impregnation (Fischbach and Dittrich, 1989) have since also been seen both from reporter driven GFP expression and EM reconstruction (Meinertzhagen *et al*., 2009). It seems likely that these three-way comparisons will also be valid in many other cases, we may even hope all. Confocal and especially ssEM reconstructions provide much richer information on cell arborizations because they can be

viewed from all possible angles, and they confirm that the neuron in question is morphologically distinguishable from others of different classes identified using the same methods. The fact that a reporter line identifies a cell type first recognized from Golgi impregnation also reassuringly corroborates the decision first made by a human observer, arguing powerfully that this successfully captures a genetic decision made by the fly in assembling its nervous system.

D. Inferring connectivity by proximity: whether juxtaposition argues connection

To state the obvious: synapses can only form when partner neurons actually contact each other. The difficulties associated with knowing first whether contact has occurred, and second whether synapses form at sites of such contact have traditionally been hard to resolve. Faced with the anonymity of neurons in the mammalian cortex, past analyses have taken a volumetrically statistical approach, with one specific proposal, the so-called Peters' rule (Braitenberg and Schüz, 1991), resting on the assumption that pre- and postsynaptic elements connect according to the numbers in which they are present in the neuropil, synapses forming in proportion to the extent of overlap between the two. In insect brains, Cajal used overlap between the profiles of Golgi impregnations to make equivalent predictions about neuropile strata, and thereby made important early contributions to the identification of relay circuits (e.g. Cajal and Sanchéz, 1915) although not incidentally in *Drosophila*. His conclusions rested upon rigid stratification in the arborisations made by specific types of neurons, and he arrived at his conclusions by matching the depth relations between the terminals of input neurons and the dendrites of their presumed targets. This matching required that the terminals of the former provide input to the dendrites of the latter, according to the dynamic law of polarization (Cajal, 1891; van Gehuchten, 1891). The latter may be true for particular terminals or dendrites but, in *Drosophila*, EM now denies this simple dichotomy in many details. Thus terminals can be postsynaptic and dendrites presynaptic, as pointed out above. For example, in the fly's optic medulla, lamina cell L1 has dendrites in the lamina that are exclusively postsynaptic (Meinertzhagen and O'Neil, 1991) but a terminal in the medulla that while having many presynaptic sites, as predicted, is also postsynaptic (Takemura *et al*., 2008). The strategy of terminal-to-dendrite overlap has helped identify many relay circuits in the fly's optic lobe (especially in larger fly species: reviewed in Douglass and Strausfeld, 2003), the olfactory system (Tanaka *et al*., 2012) and other brain regions. In the medulla, such overlaps have been quantified by microdensitometry and used to identify major relay pathways arising from specific input neurons (Bausenwein *et al*., 1992; Bausenwein and Fischbach, 1992). Another method to identify potential synaptic partners from their proximity is to express photoactivatable GFP (PA-GFP) under the control of a Gal4 driver, focally photoactivate the arbors of neurons in an area of interest, and allow the activated GFP to diffuse to the rest of the neurons. This method has been used to trace the putative connections in a sexually dimorphic pheromone responsive circuit and in the auditory circuit (Datta *et al*., 2008; Ruta *et al*., 2010; Lai *et al*., 2012).

E. Determining connectons by light microscopy

Although proximity provides only suggestive evidence of actual connection, in favourable cases synapses can actually be marked directly by light microscopic reagents (Table 1). The larval neuromuscular junction is an obvious test bed because isolated varicosities can be viewed at high resolution in fillet preparations. T-bar ribbons have been identified in varicosities using STED and other yet more novel forms of microscopy (Kittel *et al*., 2006) in conjunction with antibody reagents directed against synaptic proteins such as Bruchpilot (Brp). Several such antibodies were first isolated from a hybridoma screen of the *Drosophila* head (Buchner *et al*., 1988), some of which are now characterized (Hofbauer *et al*., 2009). Anti-Brp, monoclonal nc82 (Buchner *et al*., 1988) labels the platform of the T-bar ribbon at both neuromuscular varicosities (Kittel *et al*., 2006) and lamina tetrad synapses (Hamanaka and Meinertzhagen, 2010). Immunopuncta correspond to only about 60% the total number of lamina synapses are labeled,

however, so that possibly not all T-bar ribbons are immunolabelled, or alternatively only the tetrads are labelled and not other synaptic classes (Hamanaka and Meinertzhagen, 2010). Different immunolabelling conditions or antibody dilutions have yet to be applied that might reveal different numbers of puncta and clarify whether some entire classes of synapse may be Brp-immunonegative, or whether all synapses are positive but only with a probability determined by particular immunolabelling parameters. Dedicated specificity tests comparing immunopuncta with EM are lacking on other systems, and are badly needed for neuropiles in which many synapses are in fact known to lack T-bar ribbons. The situation is even less clear for alternative markers of presynaptic sites using reporter reagents, for example part of the Brp protein fused to a fluorescent protein (Brp-shortCFP), which colocalizes with the endogenous BRP that is recognized by nc82 (Schmid *et al*., 2008). It remains to be seen whether a related UAS construct incorporating a fluorescently tagged fragment of Brp, which depends on endogenous Brp for localization, represents a reliable marker for all active zones in neuropiles such as the mushroom body calyx (Kremer *et al*., 2010) in which only some actually have T-bar ribbons (Butcher *et al*., 2012). Alternative constructs include the presynaptic reporters neuronal synaptobrevin-GFP (Estes *et al*., 2000) and a haemagglutinin (HA) epitope-tagged synaptotagmin (HA-*syt*: Robinson *et al*., 2002). The expression of all these constructs as UAS reagents under control of a specific Gal4 driver line can in principle provide a means to label individual presynaptic sites in a particular cell type, and certainly generates clear fluorescent puncta, but it seems to us that these still require initial validation by EM. Gao *et al*. (2008) used Tub > Gal80 > as a flip-out strategy using Flies carrying the transgenes hs-Flp, UAS-Syt-HA, UAS-mCD8GFP, Tub > Gal80 >, and *ort*-Gal4 to label individual *ort*-positive optic lobe neurons. Again, at a minimum the numbers and distributions of fluorescent puncta should first be shown to match the numbers of synapses seen from EM.

For all these cases, the clear resolution of different synapses in a densely packed neuropile, especially in the z-axis, is in our view not possible from light microscopy alone, despite widespread claims in the literature based on this assumption. Improved resolution provided by modern imaging methods, such as STED (Hell and Wichmann, 1994), pcSOFI (Dedecker *et al*., 2012), STORM (Rust *et al*., 2006) or PALM (Betzig *et al*., 2006; Hess *et al*., 2006) and their variants, all provide partial answers, but require specialized microscopes or particular neurons. Clearer validation of presynaptic puncta could in principle come from simultaneous expression of markers for postsynaptic sites, for example, by means of promoter fragments for postsynaptic receptors, but the sheer diversity of the latter will necessitate the development and deployment of a bewildering array of reagents. A promising alternative to report both pre- and postsynaptic sites of contact between partner neurons comes from the GRASP (GFP Reconstitution Across Synaptic Partners) method initially developed in *C. elegans* (Feinberg *et al*., 2008) but now successfully applied in *Drosophila* (Gordon and Scott, 2009). Complementary GFP fragments fused to transmembrane proteins in neighouring cells exhibit fluorescence at sites previously shown to lie in close membrane contact (Feinberg *et al*., 2008). Judicious choice of the transmembrane protein, such as neuroligin, expressed on pre- and postsynaptic neuron partners enables GRASP to reveal synaptic sites (Feinberg *et al*., 2008). In principle, related reagents using membrane targeted innexin constructs could also be used to identify gap junctions by light microscopy, but would first need validation against populations of gap junctions for which the numbers and locations are known from EM, such as between the terminals of photoreceptors R1-R6 in the lamina (Shimohigashi and Meinertzhagen, 1998).

It seems likely that in the future the GRASP system, or a variant, will become a key method to investigate synaptic populations. The simple number of synaptic connections between two neuron partners finds no automatic functional correlate, beyond the intuition that pathways with many synapses (up to 150 or so in the medulla: Takemura, unpublished) should be stronger and less noisy than those with few. But no simple synaptic democracy foretelling pathway

strength and fidelity from synapse number has been demonstrated and neither does synapse number correlate clearly with the qualitative precision of the connections from cell to cell (Takemura *et al*., 2011). Moreover, since the gain at a synapse reflects not only the gain of its own transmission but also the gain inherited from its own input synapse in the network, the power of a synaptic population depends on where within a network each synapse acts. Thus feedback synapses are always fewer than input synapses, e.g. in a ratio of 3.88:1 for R1-R6 (Meinertzhagen and Sorra, 2001), in part because they work from the amplified signal at the latter.

On the other hand, GRASP is well qualified and possibly best suited to identifying changes in synaptic populations. Thus it is well suited to reveal natural variation in the synaptic populations borne by the same cell type. Previous EM studies reveal that such variation can be rather small in input neurons of the fly's visual system (Nicol and Meinertzhagen, 1982; Takemura *et al*., 2008), but this could be much larger in more anonymous interneurons, and especially in other systems. Alternatively GRASP can reveal changes among identified synaptic populations in mutants with altered synaptic function or specificity, and this offers a bright prospect for screening flies prior to more focused examination by EM.

GRASP may also have value in identifying synaptic circuits, but for this to happen two conditions will need to be satisfied: first, all possible combinations of neurons must be investigated to reveal those which are synaptic partners, since for reasons given above not all synaptic contacts can be predicted from neuron shape alone; and, second, contacts identified by GRASP will need to be confirmed by EM. A further feature of the GRASP system lies in its ability to distinguish the presynaptic and postsynaptic sites of contact at a synapse, and thus to reveal the direction of transmission. Against these advantages, must be set concern at the possibility that GRASP itself may alter the numbers or synapses formed between particular combinations of neurons, for which again EM validation is required.

II. ASSIGNING FUNCTIONS TO STRUCTURAL NETWORKS

Structural connectomics reveals possible pathways of information flow, the direction of signalling within those pathways from the structural distinction between pre- and postsynaptic sites, and the numerical and geometric properties of the connections between identified neurons. Collectively, these provide a starting point for dissecting the functions of neural circuits. However, a connectivity diagram lacks all information in the temporal domain, and additionally offers no insights into the biophysical and biochemical properties of its synaptic connections. These are critical determinants of the signal transduction and information transfer through synaptic connections and knowledge of their characteristics will be essential for us to understand the mechanisms of neural computation and behaviour. Electrophysiological recordings, in combination with pharmacological agents that inhibit specific channels or receptors, have so far provided the main avenue to obtain such information. But these approaches are circumscribed in *Drosophila* by the few large neurons that can serve to read out circuit function electrophysiologically, and by the specificity of vertebrate pharmacological reagents acting at *Drosophila* synapses. Since the release of the complete *Drosophila* genome, comparative genomic analyses have generated a comprehensive list of synaptic components, including transmitter receptors and ion channels (Littleton and Ganetzky, 2000; Brody and Cravchik, 2000). Most classes of receptors and ion channels identified in vertebrates are represented in flies, albeit with only one or at any rate fewer members than their vertebrate counterparts. It would appear that flies predominantly use alternative splicing, rather than gene duplication, to generate diversity (Littleton and Ganetzky 2000). Thus, compared with a mouse, it should be easier to make a fly completely devoid of a class of ion channel and to determine the functional consequences of that loss. Combined anatomical and molecular genetic

approaches now identify many functional components of synapses, and localise these to specific connections, enabling us to infer not only the sign of synaptic transmission, whether sign-conserving or inverting, but also the corresponding input/output function. In this way, information on the morphologies and synaptic contacts of different neurons lays the groundwork to simulate neural network dynamics using realistic models, providing testable predictions of network function.

Most important, targeting distinct functional components of the network by genetic means will allow us to manipulate synaptic and intrinsic firing properties in very specific ways, thereby bridging between synaptic physiology and structural connectomics.

A. Functional components determining synaptic and intrinsic properties

To bring an anatomical wiring diagram to life, then, we have to insert functional information about the transmission within and between its elements. This in turn requires information on the neurotransmitter used at each of the network's synapses and the receptor subtypes that generate postsynaptic signals, requirements that are no less demanding to ascertain than are those to generate the connectome in the first place. Not only is the identity of the neurotransmitter released often ambiguous or does it sometimes involve co-release, often of a neuropeptide with a classical fast neurotransmitter, but postsynaptic receptors also fall into a plenitude of families and subtypes that can diversify the range of signals resulting even from a single neurotransmitter.

Immunohistochemical and functional studies have long indicated that *Drosophila* shares most neurotransmitter and neuropeptide systems with vertebrates (e.g. Buchner, 1991; Nässel and Winther, 2010). The three major fast neurotransmitters, glutamate (Glu), gamma-aminobutyric acid (GABA), and acetylcholine (ACh), predominate, the latter being far more widely distributed than in vertebrate brains; others include dopamine, serotonin, histamine, aspartate, and taurine (Buchner *et al*., 1986; Kitamoto *et al*., 1998; Brotz *et al*., 2001; Bicker *et al*., 1988; Sinakevitch and Strausfeld, 2004; Kolodziejczyk *et al*., 2008; Meyer *et al*., 1996; Nässel *et al*., 1988; Schurmann *et al*., 1989; Hardie, 1987; Pollack and Hofbauer, 1991; Schafer *et al*., 1988, Restifo and White, 1990; Table 2.1). Flies also use two amines specific to protostomes, octopamine and tyramine (Busch *et al*., 2009; Monastirioti *et al*., 1995; Nagaya *et al*., 2002), which serve functions analogous to those of noradrenaline in vertebrates (Roeder, 1999). They also have a rich repertoire of neuropeptides, hormone peptides and protein hormones encoded by at least 42 genes and these additionally mediate a diverse range of slow functions (reviewed in Taghert and Veenstra, 2003; Nässel and Winther, 2010), acting broadly or systemically at a distance from their release site, by means of volume transmission (Agnati *et al*., 1995). Insofar as their wire-less mode of signaling is not revealed by a connectome, they will not be further considered here, but are of course a fundamental qualifier to network interactions for which there is such an anatomical representation.

While knowledge of the neurotransmitter released at particular synapses may provide initial evidence for the polarity and dynamics of signaling in a network of neurons, the identity of the postsynaptic receptor species at which the neurotransmitter acts, whether ionotropic or metabotropic, provides far more fertile evidence, because it defines the kinetics and mechanism of synaptic transmission (Table 2.2). Ligand gated ionotropic receptors exist as homomers or heteromers and the composition of their subunits determines their fast, ion-selective mechanism and pharmacological properties. Metabotropic receptors are monomeric G-protein coupled receptors (GPCR) that act via secondary messengers to regulate ion channel functions, exciting or inhibiting depending on the signalling pathways and ion channels they regulate. They act pre- or postsynaptically with slower kinetics than ionotropic receptors. Vertebrate transmitter receptors are classified based on their agonist responses and sequence homology. While

homologues for most receptor classes are found in flies, they may not confer the same pharmacological properties as their vertebrate counterparts. Regardless, many fly receptors and channels are known targets for neuroactive insecticides, which provide alternative means for manipulating receptor activity (reviewed in Raymond-Delpech *et al*., 2005).

Ionotropic glutamate and acetylcholine receptors appear to mediate most forms of excitatory synaptic transmission, by means of the Na^+ and Ca^{2+} conductance changes they generate. The excitatory ionotropic glutamate receptor family in *Drosophila* has approximately 30 members, divided into subfamilies based on sequence homology to vertebrate NMDA-, AMPA-, and kainate-type receptors. In flies, NMDA receptors are required in the mushroom and ellipsoid bodies for memory formation and consolidation (Wu *et al*., 2007; Xia *et al*., 2005; Tabone and Ramaswami, 2012; Miyashita *et al*., 2012). Recently, a large family of ionotropic receptors, distantly related to the ionotropic glutamate receptors, has been identified. While many members function as co-receptors for odorant receptors in the antennae, some are expressed in the brains and might serve there as ionotropic glutamate receptors (Abulin *et al.,* 2011). Nicotinic acetylcholine receptors are pentamers comprising α and β subunits; receptor diversity is further increased by combinatorial assembly of subunits as well as alternative splicing, RNA-editing and posttranslational modifications (reviewed by Jones and Sattelle, 2010). nAchRα7 is abundantly expressed in the CNS and mutant analyses reveal that nAchRα7 is required for giant fiber-mediated escape behaviour, presumably by mediating cholinergic interneuron input to dorsal lateral muscle motor neurons (Fayyazuddin *et al*., 2006). The giant fibre system provides a particular opportunity for functional connectomics because the functional contributions of elements in the pathway can so readily be assayed from its behavioural output.

GABA appears to be the major inhibitory neurotransmitter in flies. Its three known ionotropic ($GABA_A$) receptors mediate increased chloride currents and are therefore inhibitory. The most common, Rdl, was identified via mutant resistant to the insecticide dieldrin (ffrench-Constant *et al*., 1993), and inhibits olfactory associative learning (both appetitive and aversive) in the mushroom body (Liu *et al*., 2007). In addition to $GABA_A$ receptors, flies have two glutamate-gated chloride channels sensitive to ivermectin and, related, two unusual histamine-gated chloride channels (HisCl). HisCl channels, especially HisCl2 (Ort), are required in the second-order interneurons of the visual system to signal photoreceptor histamine release (Gengs *et al*., 2002; Witte *et al*., 2002; Zheng *et al*., 2002;). *ort*-Gal4 driver lines have proved effective reagents in the genetic dissection of photoreceptor inputs to visual behaviour, in particular the functional analysis of R7-mediated UV phototaxis (Gao *et al*., 2008).

Compared with ionotropic receptors, much less is known about fly metabotropic receptors. Flies have two identified glutamate-gated metabotropic receptors (mGluR) (Eroglu *et al*., 2003). DmGluRA acts via PI3 kinase, as an autoreceptor with a negative feedback action, at presynaptic terminals of motor neurons (Howlett *et al*., 2008; Lin *et al*., 2011). DmGluRA is widely expressed in the CNS but the functions of neither of the fly's two mGluR's in the CNS is clear (Ramaekers *et al*., 2001; Devaud *et al*., 2008). The functions for the two muscarinic ACh receptors are likewise not known. Metabotropic GABA receptors control the gain of olfactory neurons at the level of individual antennal lobe glomeruli (Root *et al*., 2008). Dopamine, octopamine, serotonin, and tyramine, through their corresponding G protein-coupled receptors (Roeder, 1994; Reale, 1997; Table 2.2) modulate processing within neural circuits and alter the fly's intrinsic state, presumably by volume transmission (Agnati *et al*., 1995) that leaves no anatomical trail. Thus the dopamine receptor DopR is required in the central complex for the appropriate state of the fly's arousal (Lebestky *et al*., 2009), while serotonin modulates diverse behaviours involving the fly's central state, including sleep, circadian rhythms, and olfactory learning, also through distinct receptors (Yuan *et al*., 2006; Lee *et al*., 2011). To model these actions accurately will require not only information on the exact distribution of the particular

receptors, but also the spatiotemporal features of modulator release, as well as of extracellular tortuosity (Nicholson and Sykova, 1998). These lie beyond both the scope of this review, and the current state of knowledge for fly neuropiles. Of prospective note, however, it should be possible to map the extracellular tortuosity of neuropile from the same ssEM datasets as those used to map the synaptic connections between neurons.

While the neurotransmitter receptors that determine synaptic properties may arguably be the most important variables in network function, additional components, in particular ion channels, their auxiliary subunits, and ion transporters, also govern intrinsic neuronal excitability. Voltage-gated Na^+ and Ca^{2+} channels determine the propagation of action potentials, while voltage-gated Cl^- and K^+ channels regulate ion homeostasis and excitability. Of these, K^+ channels constitute the largest and most diverse ion channel family, with about 30 members (Wei *et al*., 1990; Salkoff *et al.,* 1992; Wei *et al*., 1996). The Kv family, such as Shaker, is involved in action potential repolarization, while calcium-gated K^+ channels regulate cell excitability and action potential waveform. Until now, most channels were originally identified from hypomorphic alleles and have been analyzed in whole-fly mutants. Deciphering their roles in specific cell types will be a major challenge in the near future, one that can be confronted by approaches employing targeted genetic knockdown (Nagel and Wilson, 2011). Ion transporters and antiporters have traditionally been viewed as passive components of membrane homeostasis. However, recent evidence suggests instead that Na^+/K^+ ATPase can function to integrate spike activity and interact with K^+ conductance to provide a short-term cellular memory of previous activity (Pulver and Griffith, 2010). Furthermore, auxiliary subunits of ion channels, such as Slob for the K^+ channel Slowpoke, regulate channel activity and synaptic transmission (Ma *et al*., 2011). These higher-order functions of membrane effector molecules add a further layer of modeling complexity to the network functions of a connectome.

B. Assigning functional components to specific neurons and synapses

Assigning transmitters and receptors to specific synaptic connections is a crucial step in modelling an anatomical network. Highly specific antibodies have been raised to many neuropeptides in *Drosophila* (Nässel, 2002; Nässel and Winther, 2010), and some also recognise with great specificity fast neurotransmitters, such as GABA or histamine, and so provide reliable immunohistochemical evidence of neurotransmitter localization to specific neurons (e.g. Sinakevitch and Strausfeld. 2004; Kolodziejczyk *et al*., 2008). Such cases reveal the presence of a specific neurotransmitter and thus the likely neurotransmitter that is released, but immunolabelling for many other fast neurotransmitters is more problematic. Either no reliable antibody exists, as for acetylcholine, or the ones that exist may not distinguish between the neurotransmitter and a common metabolite, such as glutamate, or may fail to distinguish between two neurotransmitters with closely related structures. Distinction between octopamine and tyramine has often been controversial, for example, and the balance between the two neurotransmitters may shift as the result of handling (Kononenko *et al*., 2009). In such cases and in the absence of reliable immunolabelling evidence, however. many fast neurotransmitters have been identified only indirectly by neuronal expression of the corresponding enzymes for their biosynthesis or of vesicular transporters (Table 2.1). The case for a particular neurotransmitter phenotype can obviously be strengthened by using antibodies directed against both the biosynthesis enzyme and the transporter and observing their co-localisation to the same neurons. The same considerations apply to receptor and ion channel expression as to neurotransmitters, and in all cases the signal is often distributed in neurites or terminals densely packed in the neuropile, and requires high resolution to locate. Even with a strong immunosignal, immunohistochemistry alone seldom has sufficient resolution to discern individual neurons in their entirety. The best cases come from single neurons with a wide-field arbor, but these are typical of neuromodulatory rather than relay neurons however. In situ

hybridization, an alternative, may be used to identify the cell bodies of neurons that express genes for particular transmitters (e.g. Barber *et al*., 1989; Okada *et al*., 2009), but typically lacks resolution and labels the cell's nucleus not its neurites.

Given the capriciousness of immunolabelling, alternative reporters have been widely used. In particular, promoter constructs and enhancer trap based Gal4 drivers (or other two-part expression systems) have been used extensively to identify neurons that express genes of interest (reviewed in Duffy, 2002; Table 2.1). For example, the promoter fragment of the gene Cha (for choline acetyltransferase) when fused to Gal4 to generate the Cha-Gal4 transgene (or driver) drives GFP to identify putative cholinergic neurons (Salvaterra and Kitamoto, 2001; Raghu *et al*., 2011). To facilitate identification of neuron types, genetic mosaic methods, such as the MARCM (Lee and Luo, 1999) and "flip-out" techniques (Wong *et al*., 2002), are often used to label neurons singly or in small numbers to reveal their three-dimensional forms (Raghu and Borst, 2011; Raghu *et al*., 2007, 2011). In addition to revealing gene expression patterns, this approach provides a convenient way to manipulate and view the neurons, especially at points of their synaptic input or output, for comparison with ssEM (Gao *et al*., 2008). However, whether a driver faithfully captures the expression pattern of the corresponding gene is uncertain. The same promoter construct inserted in different genomic locations gives rise to distinct expression patterns because of the actions of nearby enhancers. To mitigate such positional variegation effects, transgenes can be inserted into a specific genomic location using the ϕC31-mediated transgenesis system (Bischof *et al*., 2007, Pfeiffer *et al*., 2008). Indeed, large collections of promoter Gal4 drivers using promoter fragments have in this way been generated in the Janelia screen, above (Pfeiffer *et al*., 2008). Even so, the extent of the promoter region in the genome is in any case often unclear. Based on sequence conservation, comparative sequence analysis of 12 *Drosophila* genomes has been used to identify enhancer elements and assist in the design of promoter constructs (Odenwald *et al*., 2005; Gao *et al*., 2008). Based on few systematic analyses, enhancers appear to be organized in blocks of conserved sequences, each of which captures only a part of the entire expression pattern and none of which captures all (Kuzin *et al*., 2012). These are not trivial issues. For example it might be thought straightforward to identify the neurotransmitter phenotype of an identified class of *Drosophila* neuron based simply on the Gal4 expression pattern driven by a corresponding promoter fragment of the appropriate gene. In practice, Gal4 lines do not invariably recapitulate those for neurotransmitter antibody labeling, leaving doubt as to which evidence is the more reliable and whether neurons exclusively express a single fast neurotransmitter. Thus, two lamina neurons L3 and L4, but not two medulla centrifugal cells, C2 and C3, express under the control of a vGAT-Gal4 line, compatible with being GABA sequestering and thus possibly GABAergic (Raghu *et al*., 2012), whereas from immunocytochemistry C2 and C3 are GABA positive and L3 and L4 are not (Kolodziejczyk *et al*., 2008).

The most reliable, albeit labour-intensive, method to recapitulate an endogenous expression pattern is to insert (or knock-in) Gal4 into appropriate locations in the corresponding genomic locus, by means of homologous recombination (Rong and Colic 2000; Demir and Dickson, 2005). An alternative is to use the MiMIC (Minos-mediated integration cassette) system, a versatile genomic engineering system that converts transposons into gene- or enhancer-traps via ϕC31 recombinase-mediated cassette exchange (Venken *et al*., 2011). Thousands of MIMIC transposon insertions have been generated, enabling modifications of the targeted loci. In addition to modifying endogenous loci, Gal4 could be knocked into a large (30~100kb) genomic DNA fragment, such as a BAC (bacterial artificial chromosome) clone, that contains the gene of interest as well as most, if not all, of its relevant enhancers. The Gal4-containing BAC clones can then be reintroduced into the genome via the ϕC31-mediated transgenesis (Venken *et al*., 2006; Venken *et al*., 2009; Chan *et al*., 2011). If the locations of enhancers or the translation initiation sites are not evident from the sequences, it might be desirable to fuse

the Gal4 gene to the end of the coding region to generate a "bi-cistronic" gene. While flies lack effective IRES (internal ribosome entry site) sequences, viral T2A peptide, which has a “ribosomal skipping” property, has been exploited to generate Gal4 in-frame fusion with the gene of interest (Diao and White, 2012). In these ways, state-of-the-art transgenesis approaches may reproduce almost any gene expression pattern. Powerful as these are, however, none will reliably repeat the cell-specific patterns of alternative splicing, which generate particular receptor and channel variants having functionally different properties. It is the latter that we need to insert into connectome data, and the requirements to generate them must await further development of new methods.

What alternatives to immunolabelling and reporter expression exist to reveal transmitter or channel phenotypes? Once a highly specific driver that marks a specific neuron type of interest is available, profiling that cell’s transcripts is the most direct way currently available to secure its electrophysiological signature, using the transcripts and their spliced variants to gain insight to the molecular basis of activity in the neuron of interest. Simple as it appears, the challenge is to find a method to isolate a homogeneous population of cells in quantities sufficient for robust signal detection. Many methods have been developed. Cell purification methods, such as FACS (fluorescent activated cell sorting) and MACS (magnetic-activated cell sorting), have been used extensively but are frequently plagued by problems of incomplete cell dissociation and/or isolation (Neves *et al*., 2004; Zhan *et al*., 2004; Yonekura *et al*., 2006). Manual sorting, while alleviating the problems of contamination and resolving transcripts at the single-cell resolution, is low in yield and therefore only suited to examining a limited number of transcripts (Neves *et al*., 2004; Takemura *et al*., 2011). However, cell-to-cell variations, which could be of significant functional consequence, can only be captured by single-cell methods (Schulz *et al.,* 2006; Goaillard *et al*., 2009; Marder, 2011). The TU-tagging method isolates from bulk cellular RNA newly synthesized RNA that has been modified with a uracil analogue in a cell-specific fashion (Miller *et al.,* 2009). This method avoids tedious cell dissociation and isolation methods but for the same reason as for FACS or MACS it is difficult to estimate the level of purity. A promising method, called INTACT (isolation of nuclei tagged in a specific cell type), marks and isolates the nuclei of a specific cell type with a genetically encoded tag (Henry *et al*., 2012; Steiner *et al*., 2012; Bonn *et al*., 2012). In addition to profiling gene expression with RNA-sequencing analysis, it could be used profile chromatin modification using ChIP-sequencing analysis. Just as an ultimate objective for the neurobiology of *Drosophila* is to construct the connectome of an entire region of the brain, identification of the transcriptome of each of the component neuron classes will be required to add to that connectome the full repertoire of functional information.

Finally, functional connectomics urgently requires means to localize molecular determinants of synaptic transmission, especially postsynaptic receptors, to specific synapses. Active components, such as voltage-gated channels, present on axons and dendrites are known to shape signal prorogation and enable complex neural computation (reviewed in Kress and Mennerick, 2009; London and Hausser, 2005). For most fly neurons, information on the subcellular localization of these components is not available and modelling their electrophysiological behavior has been based on passive membrane properties (Gouwens and Wilson, 2009). Immuno-EM methods have until recently been the sole option to localise receptors and channels to their subcellular locations. Some progress has been made with these at neuromuscular (e.g. Sone *et al*., 2000) and photoreceptor (Hamanaka and Meinertzhagen, 2010) synapses, but the methods are individual and technically demanding. Novel approaches to examine the expression of synaptic proteins, especially postsynaptic receptors, are badly needed as a complement. In particular these are needed to locate the expression sites for receptor proteins identified from the transcript profiles of single identified neurons (e.g. Takemura *et al*., 2011), but immuno-EM attempts simply prove unsuccessful for

most combinations of antibody and fixation conditions. Genetic approaches provide several possible alternative solutions. In an ideal approach, synaptic proteins could be genetically tagged with a non-perturbing label that can be visualized by optical means in living cells and also by EM, preferably applied consecutively (Gaietta *et al*., 2002). Epitope tagged constructs are useful for light microscopy (Jarvik and Telmer, 1998), but most epitope tags (HA, His etc) lose immunoreactivity after fixation for EM, and applications at EM level are as a result rarely reported or may require cryo-EM methods beyond the reach of most laboratories. Additional constructs that could withstand fixation for EM are therefore greatly needed.

As alternatives to antibody based methods, transgenically encoded recombinant proteins incorporating a tetracysteine motif CCPGCC can bind to, and induce fluorescence in, non-fluorescent membrane-permeant biarsenical derivatives either of fluorescein, FlAsH-EDT_2, or a comparable derivative of the red fluorophore resorufin, ReAsH-EDT_2, causing these to gain fluorescence. The fluorescence of ReAsH after binding can photoconvert DAB to yield with osmium an electron-dense reaction product that is visible in EM (Gaietta *et al*., 2002). FlAsH has been used successfully in *Drosophila* at the neuromuscular junction of larval fillet preparations (Marek and Davis, 2002) and ReASh (Gaietta *et al*., 2002) to identify Connexin turnover in cell cultures (Gaietta *et al*., 2002), but depends critically on the chemical synthesis of the substrate and has not been successful with tissue preparations; apparently no report has yet appeared using the brain in *Drosophila*. Another transgenic approach uses a genetically encoded photosensitizer mini singlet oxygen generator (miniSOG) to generate singlet oxygen upon blue-light illumination and catalyse polymerization of DAB to generate an osmiophilic electron-dense reaction product (Shu *et al*., 2011). This system has been used to photoablate neurons in *C. elegans* (Qi *et al*., 2012), but its successful application in *Drosophila* has likewise yet to be reported.

C. Moving from molecular to electrophysiological data

Armed with knowledge of a neuron's transcriptome, in particular the few postsynaptic receptors it may express from amongst the full array of those possible, we can begin to assign channels and other electrophysiological determinants to the neuron based on its molecular signature. To do so, however, we need knowledge of the channels' properties, their respective ion selectivity, conductance, kinetics, and pharmacology. The "giant" neuron system, in combination with whole-cell patch recording and mutant analyses, has greatly facilitated the characterization of ion channels (Saito and Wu, 1991). Most recent approaches, however, focus on expressing and characterizing cloned receptors and channels in non-neuronal systems that are otherwise electrophysiologically inactive. The S2 cell line has been used as a functional expression system for a cloned muscarinic cholinoceptor, and a stable line developed (Millar *et al*., 1995). Using *Xenopus* oocytes as a heterologous expression system has revealed that the potency of the *Drosophila rdl* GABA receptor varies depending on splice-variant and stage-specific RNA editing (Jones *et al*., 2009). The choice of cell for the particular expression system is also important because the channels and receptors may be modified in the cell type that expresses them. Thus TRPL channels are constitutively active when expressed in S2 cells but silent in HEK cells (Lev *et al*., 2012). These few examples reveal the fertile opportunities facing future *in vitro* functional expression studies. Other details of the topic will not be considered further here, but will be required eventually to translate receptor and channel expression data and model these into electrophysiological signals, in the final stage of predicting a functional connectome,

III BRIDGING SYNAPTOPHYSIOLOGY TO STRUCTURAL CONNECTOMICS

To evaluate the function of a synaptic circuit requires not only a means to target its disruption to specific neurons or synaptic components in the network but also some form of functional assay

for the outcome of that disruption. The two most obvious readout modes are to monitor neural activity at electrophysiologically accessible sites or to record the change in a system-specific behavioral assay. The genetic strategies and reagents that can reproducibly disrupt transmission at, or conduction in, specific neurons, and examine the behavioural consequences, have all recently been extensively reviewed (Simpson *et al*., 2009; Venken *et al*., 2011). In the following, we will review strategies and tools for the targeted manipulation of synaptic components and the monitoring of neural activity.

A. Monitoring neuronal activity in circuits

Intracellular electrophysiological recording methods remain the gold standard for monitoring neural activity in both vertebrate and invertebrate brains. However, the small sizes of *Drosophila* CNS neurons had for many years restricted electrophysiological investigation to practitioners in a few expert laboratories until the recent application of whole-cell patch techniques (Wilson et al., 2004; Rohrbough and Broadie, 2002). While still technically challenging and with recordings limited to one or very few neurons per preparation, such electrophysiological recordings are nevertheless feasible, provide the highest sensitivity and greatest temporal resolution, and have been instrumental in decoding synaptic circuit functions in the olfactory (Wilson *et al*., 2004), visual (Joesch *et al*., 2008, 2010; Wardill *et al*., 2012), giant fibre (Augustin *et al*., 2011), and circadian (Nitabach *et al.,* 2006; Sheeba *et al*., 2008) systems. In particular, the use of genetic tools to label cells with GFP as an aid in guiding recording electrodes and in activity manipulation has greatly assisted these methods, helping to establish the functional connectivity of circuits and decrypt sensory codes (Olsen and Wilson, 2008a,b; Tanaka *et al*., 2009).

Functional imaging has the advantage, but also the disadvantage, of monitoring the activity of many neurons simultaneously. Several genetically encoded activity reporters have been applied in flies. Synapto-pHuorin, a pH-sensitive fluorescent protein coupled to synaptobrevin has been used to monitor synaptic vesicle fusion events (Ng *et al*., 2002) and so record transmission between neurons in the antennal lobe. Most genetically encoded activity indicators use intracellular calcium as a proxy for neuronal activity, however. Ratiometric or FRET (Förster resonance energy transfer)-based Ca^{2+} indicators, such as TN-XXL, which because they cancel out correlated signals in both channels, such as those arising from the animal's own movements, are particularly well suited for recording neural activity in behaving animals (Mank *et al*., 2008). GCaMP and its derivatives, which are based on circularly permutated GFP, have been most widely used because of their high sensitivity (Nagai *et al*., 2001; Tian *et al*., 2009). Calcium indicators are constantly evolving, frustrating any useful current summary, however, and the next generation of GCaMP derivatives have both significantly improved sensitivity and temporal resolution, and spectra that are extended into the red by the incorporation of new fluorescent indicators (Zhao *et al*., 2011). While individual spikes cannot be resolved directly by calcium indicators, several methods that depend on deconvolution or other model-fitting techniques have been used to infer timing and pattern of spikes based on the calcium signal observed (Holekamp *et al*., 2008; Vogelstein *et al*., 2009). Alternatively, fast 2-photon random access scanning microscopy now provides millisecond resolution sufficient to interrogate the functionality of individual synaptic circuits after single-cell activation (Katona *et al*., 2012), although this advance has yet to be reported in *Drosophila* studies.

On a longer time scale (hours or days), in what was an early approach to develop an activity stain in the fly's brain, [^{3}H]-2-deoxyglucose uptake was used to monitor neural activity and identify the key brain regions and neurons required to process distinct visual stimuli (Bausenwein *et al*., 1990; Bausenwein *et al*., 1994). Ultimately the method was limited in its application by poor spatial and temporal resolution, by its failure to discriminate neuronal from

glial activity, and because it is only an indirect metabolic proxy of electrical activity. The translocation of CaMKII (calcium/calmodulin-dependent kinase II) mRNA to postsynaptic sites and its local translation could be used instead as a surrogate for neural activity (Ashraf *et al*., 2006). To monitor sustained activity in specific neuron classes, CaLexA (calcium-dependent nuclear import of LexA), has been developed. This method uses the activity-dependent nuclear import of a chimeric transcription factor, LexA-VP16-NFAT (nuclear factor of activated T cells) to convert neural activity into LexA-dependent GFP expression (Masuyama *et al*., 2012). In another method, to detect the release of neuromodulators such as dopamine, and their action sites, a method called DopR-TANGO has been developed (Inagaki *et al*., 2012). This experimental strategy, originally demonstrated in culture cells, converts a transient receptor-protein interaction into reporter expression (Barnea *et al*., 2008). In the case of dopamine, activation of a chimeric receptor (a dopamine D1 receptor fused to the transcription factor LexA) recruits the signalling protein arrestin1 fused to the TEV (tobacco etch virus) protease, which cleaves and releases LexA to activate reporter expression in the nucleus. An alternative approach, in which an indicator or "sniffer" for glutamate has been developed to monitor extrasynaptic glutamate and its dynamics, but this method has yet to be applied in flies (Okubo *et al*., 2010). For neuropeptides, Epac1-camps, a genetically encoded FRET-based cAMP sensor, has been used to monitor the action of the neuropeptide pigment dispersing factor, PDF, on its GPCR receptor (Shafer *et al*., 2008).

B. Targeting specific synaptic components

As part of wide mission to dissect neural function using genetic means, many techniques have been developed over the years to excite or inhibit activity in genetically identified neurons. Methods for inhibiting neuronal activity include the use of tetanus toxin or a dominant-negative form of dynamin GTPase to block chemical synaptic transmission (Sweeney *et al*., 1995; Kitamoto, 2001) as well as of light-driven halorhogopsin pump to increase Cl^- influx (Inada *et al*., 2011). To excite neurons, channel rhodopsin and TrpA1 channels have both been used to increase cation influx using either light or temperature as a trigger. Chronically exciting or inhibiting neurons has also been achieved by overexpressing Na^+ and K^+ channels, respectively (White *et al*., 2001; Baines *et al*., 2001; Nitabach *et al*., 2002; Hodge *et al*., 2005). These techniques allow targeted manipulation of neuronal activity, and have been extensively reviewed (Venken *et al*., 2011). In the following, we will focus instead on methods that target the function of specific synaptic components in genetically identified neurons.

Given that most synaptic components have pleiotropic functions, their contributions in neural circuits are difficult to dissect using traditional genetic approaches. Currently, RNAi (RNA interference) targeted by the Gal4/UAS expression system is the most straightforward way to affect neuron function in genetically identified circuits. Several genome-wide RNAi libraries targeting essentially every fly gene have been generated (Dietzl *et al*., 2007; Ni *et al*., 2009; Ni *et al*., 2011). Notably, short-hairpin RNAs (shRNA) may be used to target specific exons, allowing the function of alternatively spliced variants to be studied. The following examples illustrate how RNAi approaches have been used to manipulate neural activity: RNAi has been used to inactivate neurons by knocking down the Ca^{2+} channel cacophony or the Na^+ channel para (Worrell and Levine, 2008; Zhong *et al*., 2010) as well as to activate neurons by knocking down the Shaw K^+ channel (Hodge and Stanewsky, 2008). Related, RNAi-mediated knock down of NMDA receptors has been used to differentiate two forms of NMDA-dependent memory processing, in the mushroom body and the ellipsoid body (Wu *et al*., 2007).

Despite its power and convenience, the RNAi approach is not without pitfalls. First, it almost always generates hypomorphic phenotypes because knock out is incomplete, although this could be improved by introducing Dicer-2 enzyme or additional RNAi transgenes (Dietzl *et al*., 2007). However, the extent of RNAi knock down and the level of remaining transcripts are

rarely quantified, complicating interpretation of the outcome of their loss. Another pitfall of RNAi is the so-called "off-target" effect, which knocks down transcripts other than those intended. To offset this problem, RNAi target specificity can be validated by rescuing the phenotype with an RNAi-resistant transgene generated using an alternative codon or a cDNA from another *Drosophila* species (Schulz *et al*., 2009; Kondo *et al*., 2009; Langer *et al*., 2010).

Many channels, such as the K^+ channel Shaker, contain multiple pore-forming subunits, so that expressing a truncated version of the subunit should block their functions (Gisselmann *et al*., 1989), providing a means to disrupt these. This dominant-negative strategy has been applied to other K^+ channels, such as Eag and Shaw (Broughton *et al*., 2004; Hodge *et al*., 2005), as well as to the Na^+/K^+ ATPase (Sun *et al*., 2001; Parisky *et a*l., 2008). Membrane-tethered toxins (t-toxins) provide a valuable alternative for cell-autonomous modulation of channels and receptors (reviewed in Ibañez-Tallon and Nitabach, 2012). For example, four spider toxins tethered to membrane with a glycosylphosphatidylinositol (GPI) anchor have each been shown to block their previously identified targets, including Ca^{2+}, K^+ and Na^+ channels (Wu *et al*., 2008). As for *in vivo* application, blocking the inactivation of the voltage-gated Na^+ channel para in the fly's circadian system clock neurons using a GPI-tethered d-ACTX-Hv1a toxin induces rhythmic action potential bursts and depolarised plateau potentials, causing circadian phase advancement (Wu *et al*., 2008). Interestingly, Sleepless, a Ly-6/neurotoxin family member, is an endogenous regulator of Shaker K^+ channels (Wu *et al*., 2010) while other members of the Ly-6 family are endogenous modulators for nAChR in insects and mammals (Choo *et al*., 2008; Morishita *et al*., 2010). Comparing RNAi and dominant-negative approaches, which intervene at the stages of channel synthesis and assembly, t-toxins directly bind and block channels and receptors after these are assembled, suggesting that they may have faster kinetics. In addition, they appear to be functionally inert in cells that lack genetic targets.

C. Towards the reprogramming of neural activity

With the range of tools now available for modifying neurons, either their intrinsic excitability or their synaptic properties, the means to reprogram neural activity are readily available that underlie applications aimed at deciphering mechanisms of neural computation. Astute application of those tools has in fact already advanced our understanding of olfactory and visual circuit functions (reviewed in Wilson, 2011; Borst and Euler, 2011). In choosing appropriate genetic strategies, however, it is still important to consider how each reagent might affect the complex dynamic electrophysiological behaviors of the neurons under manipulation (Koch, 1998). While three different K^+ channels have been used as electrical shunts to inhibit neural activity, for example, these have different strengths and effects (Holmes *et al*., 2007). Kir2.1 and dOrk-deltaC are both inwardly rectifying K^+ channels and depolarise resting potential, while EKO, a truncated version of the Shaker voltage-sensitive K^+ channel, shortens action potentials by speeding up repolarisation. Both dTRPA1 and ChR2 have been widely used to excite neurons using temperature and light, respectively, to trigger their actions, but they produce rather different effects on larval motor neurons (Pulver *et al*., 2009). Thus, dTRPA1 but not ChR2 expression in motor neurons eliminates adaptation in spike frequency and produces abnormal spiking patterns. Blocking the Na^+/K^+ ion pump with a dominant-negative strategy specifically abolishes afterhyperpolization and reduces spike frequency while preserving the overall spiking pattern (Pulver *et al*., 2010). Furthermore, high-level expression of neuron activators, such as NaChBac, can lead to inhibition (Luan *et al*., 2006). These examples collectively underscore the advantages of having multiple means to modulate intrinsic components, and the need to monitor the activity of the manipulated neurons carefully for purposes of informed comparison.

Manipulating neural activity also needs to confront the intrinsic adaptability of individual neurons and circuits. Similar electrophysiological behaviour can be achieved by very different

combinations of underlying properties in the neural circuits (reviewed in Marder, 2011; Turrigiano, 2008). Examples drawn from both vertebrate and invertebrate brains reveal how readily homeostatic mechanisms can compensate for perturbations in neural excitability (Marder and Goaillard 2006; Turrigiano and Nelson, 2004; Nerbonne *et al*., 2008). In flies, homeostatic regulation has been well studied at the neuromuscular junction (Stewart *et al*., 1996; Bergquist *et al*., 2010) but little analysed in the CNS. In larval motor neurons, the mRNA level of the Na^+ channel para is negatively regulated by increased excitability (Mee *et al*., 2004). In an interesting contrast, however, *Drosophila* flight motor neurons lack obvious homeostatic regulation and RNAi-mediated knock down of the K^+ channels Shaker and Shal reduces total current amplitudes to a level similar to that observed with pharmacological strategies (Ryglewski and Duch, 2009). Even though the extent of homeostatic compensation elsewhere in the fly's CNS is not currently clear, it would obviously be prudent to avoid chronic knock down of channels and receptors, especially through pupal development when adult circuits are forming. Quantification of the extent of knock down and comparisons of the effects produced by different reagents, are both precautions that come most obviously to mind, as is awareness of potential compensatory mechanisms. A potentially powerful strategy to overcome such compensation is to modify ectopically expressed channels and receptors so that they are sensitive to unique transient pharmacological modulation (Wulff *et al*., 2007). This combination of molecular biology and pharmacology has yet to be applied in flies however.

IV. CONCLUSIONS

Based on very few precedents in *Drosophila*, most in the visual system (e.g. Meinertzhagen and O'Neil, 1991; Takemura *et al*., 2008), opportunities to undertake connectomics studies using ssEM approaches are still clearly nascent. To this extent, any experience can only be viewed as premature, but even so certainly supports a number of preliminary conclusions: a) that network complexity is simply huge but connections far from random; b) that pathway strength, as reported by numbers of synaptic contacts, varies widely, and that transmission from each site diverges to multiple postsynaptic elements, often about four; and c) that sensory input pathways are not strictly segregated, but that motor pathways have yet to be characterized. The prospects for future studies using an approach at EM level, although heavily circumscribed by current technical problems, are nevertheless bright and a major endeavour especially at the Janelia Farm campus of Howard Hughes Medical Institute. Surrogate approaches using genetic approaches, especially GRASP (Feinberg *et al*., 2008; Gordon and Scott, 2009), are currently being pursued in several labs and seem destined to add numerical confirmation to ssEM approaches, once their validity is fully confirmed by the latter. Findings from either approach are circumscribed by the possibly doubtful status of pathways with few synaptic contacts, the possibility that these might show activity-dependent regulation (Yuan et al., 2011), or other forms of plasticity, and the lack of knowledge concerning the synaptic transfer characteristics at different sites.

Functional validation of circuit information relies on genetic dissection that has been tested as a proof-of-principle approach (e.g. Rister *et al*., 2007) and demonstrated to confirm novel functions for identified neuron classes (Gao *et al*., 2008), but which relies critically upon several requirements: a) the cell-specificity and strength of the driver line; b) the effectiveness and precision of functional disruption, as previously reviewed (Simpson, 2009); and c) comparisons between the outcomes derived from different reagents. Their limit lies particularly in two areas: a) the redundancy or degeneracy of pathways that can substitute for one that is blocked; and b) related, the preferred requirement to apply a blockade conditionally and then assay the recovery of behavioural function, an opportunity provided particularly by UAS-shi^{ts1}. Remedies may therefore be sought by: a) applying conditional blockade concurrently in multiple cell types or

pathways, using combinatorial systems, for which the requisite genetic reagents then become highly complex, and possibly limiting; and b) the development of new ts alleles of synaptic or ion channel genes, an approach that has been followed by the Ganetzky lab in particular (Palladino *et al*., 2002; Littleton *et al*., 1998) but which still holds further promise. It seems clear that these and related reagents will be needed to pioneer the analysis of new connectomic data, and that this need will become more pressing as the latter available becomes more voluminous.

The prospect of a strong and growing union forged between anatomical network data, and the instrumental application of genetic disruption methods will, we predict, enable the successful application of functional connectomics to specific pathways in the fly's brain. These in turn will, we eagerly anticipate, provide the sort of causal analysis of fly behaviour that has hitherto been denied to alternative functional approaches using more traditional electro- or optophysiological approaches. It is not widely accepted in the field that while such recording methods alone can reveal the dynamics of network function in brains, they do not reveal the causal basis of behaviour, but rather are its correlate. Only when we block the function of an identified neuron, or rescue that function in a genetic loss-of-function mutant, can we truly be said to have probed that neuron's function and established the causal basis of a corresponding behaviour. It is this causality that we propose is the ultimate objective of functional connectomics, one in which the chief tools to be used are, we suggest, genetic.

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Table 1. Imaging Neurons and Circuits

Organelle	Subtype	Dimensions		Imaging method	Selected reagents	Reference
		x/y (diameter)	z/length/depth			
synaptic vesicle	small, clear	5-30 nm		TEM		Meinertzhagen and O'Neil, 1991
	dense core	60-180 nm		TEM	αpeptide Ab	Nässel and Winther, 2010
T-bar ribbon	platform	<175 nm / <350 nm	<450 nm	TEM, FIB, STED	αBrp Ab (nc82); Brp-GFP	Kittel et al., 2006; Hamanaka and Meinertzhagen, 2010
	pedestal		135 x 350 nm		SUK4 (αKinesin Ab)	Hamanaka and Meinertzhagen, 2010
postsynaptic site		< 100 nm	~ 20 nm	TEM, STED, confocal	αreceptor Ab	Nicolaï *et al.*, 2010; Butcher *et al.*, 2012
soma	nucleus	2-7 μm		confocal	αElav Ab	Bier *et al.*, 1988
	perikaryon	3-10 μm		confocal	αRepo Ab	Xiong *et al.*, 1994
axon		~0.1-2 μm	<~100 μm	confocal	mCD8GFP; tau-GFP	Lee and Luo, 1999; Stone *et al.*, 2008
terminal		~0.2-7 μm	1-10 μm	confocal	Syt-GFP; Brp-GFP	Kittel *et al.,* 2006; Gao *et al.,* 2008
dendrite		50-500 nm	<~25 μm	TEM, confocal	HRP::CD2; mCD8GFP	Edwards and Meinertzhagen, 2009
neuron	cell type			confocal	mCD8GFP	Lee and Luo, 1999
	single cell				MARCM	Lee and Luo, 1999
neuropile	general	<100 μm / <100 μm	<50 μm	confocal	nc82, ab49, nc46, aa2	Hofbauer *et al.*, 2009
	specific			confocal	e.g. nb236, fb45	Hofbauer *et al.,* 2009

Imaging method		Resolution (x/y, z)	
TEM	serial-section transmission electron microscopy	2 nm, 40-60 nm	Denk et al., 2012 (their Table 1)
FIB	serial block-face scanning electron microscopy with focused ion beam ablation	<10 nm, <10 nm	
STED	stimulated-emission depletion microscopy	~30 nm, ~30 nm	
Confocal	confocal microscopy	~200 nm, ~500 nm	

Table 2.1. Major Neurotransmitters and Related Transporters and Biosynthesis Enzymes

Neurotransmitter	Gene	Synonym	CG #	Gal4 line	Reference
Acetylcholine	*Cha*	*ChAT*	CG12345	cha-Gal4	Yasuyama *et al*., 1995
Glutamate	*VGlut*	*DVGlut*	CG9887	OK371-Gal4	Mahr and Aberle, 2006
				dvGlut-Gal4	Daniels *et al*., 2008
GABA	*VGAT*	*vGAT*	CG8394		Enell *et al*., 2007
	GAD1	*gad*	CG14994	GAD1-Gal4	Ng *et al*., 2002
	b	*DGad2*	CG7811		Okada *et al*., 2009
Histamine	*Hdc*	*hdc*	CG3454		Borycz *et al*., 2005
Dopamine	*DAT*	*fmn*	CG8380	R58E02-Gal4	Liu *et al*., 2012
	ple	*TH*	CG10118	TH-Gal4	Friggi-Grelin *et al*., 2003
	DDC	*ddc*	CG10697	Ddc-Gal4	Friggi-Grelin *et al*., 2003
Serotonin	*SerT*	*dSERT*	CG4545		Giang *et al*., 2011
	DDC	*ddc*	CG10697	Ddc-Gal4	Friggi-Grelin *et al*., 2003
Octopamine	*Tbh*	*Tβh*	CG1543	Tβh-Gal4	Stowers, 2011
	Tdc2	*dTdc2*	CG30446	tdc2-Gal4	Cole *et al*., 2005
Tyramine	*Tdc2*	*dTdc2*	CG30446	tdc2-Gal4	Busch *et al*., 2009

Table 2.2 Neurotransmitter Receptors and Gap Junction proteins

Acetylcholine Receptors			
Type	Gene	Synonym	CG #
Nicotinic	*nAcRa-96Aa*	*Dα1*	CG5610
	nAcRa-96Ab	*Dα2*	CG6844
	nAcRa-7E	*Dα3*	CG2302
	nAcRa-80B	*Dα4*	CG12414
	nAcRa-34E	*Dα5*	CG32975
	nAcRa-30D	*Dα6*	CG4128
	gfA	*Dα7*	CG8109
	nAcRb-64B	*Dβ1*	CG11348
	nAcRb-96A	*Dβ2*	CG6798
	nAcRb-21C	*Dβ3*	CG11822
Muscarinic	*mAcR-60C*	*mAChR*	CG4356
	CG7918		CG7918

Glutamate Receptors			
Kainate	*GluRIIA*	*DGluR-IIA*	CG6992
	GluRIIB	*DGluR-IIB*	CG7234
	GluRIIC	*DGluRIII*	CG4226
	GluRIID	*KaiRIA*	CG18039
	GluRIIE	*GluR-IIE*	CG31201
	Clumsy	*GluR39B*	CG8681
	CG5621	*DKaiRIC*	CG5621
	CG3822	*DKaiRID*	CG3822
	CG9935	CT36399	CG9935
	CG11155	CT30863	CG11155
AMPA	*Glu-RI*	*DGluRI*	CG8442
	Glu-RIB	*DGluRIB*	CG4481
NMDA	*Nmdar1*	*dNR1*	CG2902
	Nmdar2	*dNR2*	CG33513
Cl channel	*GluClα*	*DmGluClα*	CG7535
Metabotropic	*mGluRA*	*DmGluRA*	CG11144
	mtt	*DmXR*	CG30361
	CG32447		CG32447
	pog		CG31660
	CG31760		CG31760

GABA/glycine Receptors			
$GABA_A$	*Rdl*	*GABAAR*	CG10537
	Grd	*DmGABA*	CG7446
	Lcch3	*DmGABAβ*	CG17336
	CG8916		CG8916
	CG6927	CT21430	CG6927
	CG7589	CT23187	CG7589
	CG11340	CT5896	CG11340
	CG12344	CT23391	CG12344
$GABA_B$	*GABA-B-R1*	*mGABA-B-R1*	CG15274
	GABA-B-R2	*mGABA-B-R2*	CG6706
	GABA-B-R3	*mGABA-B-R3*	CG3022
	CG3078		CG3078
	CG43795	CG34372	CG43795

Histamine Receptors		
Gene	Synonym	CG #
HisCl1	*hclB*	CG14723
ort	*HisCl2*	CG7411

Dopamine Receptors		
DopR	*dumb*	CG9652
DopR2	*DAMB*	CG18741
D2R	*DD2R*	CG33517
DopEcR	*DmDopEcR*	CG18314

Serotonin Receptors		
5-HT1A	*5-HT1ADro*	CG16720
5-HT1B	*5-HT1BDro*	CG15113
5-HT2	*5-HT2Dro*	CG1056
5-HT7	*5-HT7Dro*	CG12073
CG42796	CG8007	CG42796

Octopamine Receptors		
oa2	*DmOctβ1R*	CG6919
Octβ2R	*DmOctβ2R*	CG33976
Octβ3R	*DmOctβ3R*	CG42244
Oamb	*DmOctβ1Rb*	CG3856
Oct-TyrR	*DmOctoR1*	CG7485

Tyramine Receptor		
TyrR		CG7431
TyrRII		CG16766

GAP Junction innexins		
ogre	*inx1*	CG3039
inx2	*Dm-inx2*	CG4590
inx3	*Dm-inx3*	CG1448
inx4	*zpg*	CG10125
inx5	*Dm-inx5*	CG7537
inx6	*prp6*	CG17063
inx7	*prp7*	CG2977
shakB	*pas*	CG34358